will be cut from the blocks at 4 μm and float-mounted on adhesive (silanized) glass slides.

A standard, sensitive, generic detection system will be used with each ER-β primary antibody. This promotes both technical and cost efficiency and uniformity of results. Each antibody may require certain minor modifications for optimum performance, which usually involves adjustments of antibody concentration, incubation time, or the use of various antigen-enhancement techniques. The detection system is based on linking the primary antibodies to biotinylated secondary antibodies to peroxidase-conjugated streptavidin. The signal is developed with diaminobenzidine/hydrogen peroxide as the chromogen, which is enhanced with osmium tetroxide, and contrasted to alight methyl-green counterstain. Specifics are as follows:

Generic Detection System

1. Preparing sections

   - cut at 4μm
   - float-mount on PLUS-coated slides in an adhesive free water bath
   - dry overnight at room temperature

2. Deparaffinizing

   - graded xylene, alcohols, and distilled water

3. Antigen retrieval (depending on primary antibody)

   - none, or
   - 0.1 M citrate buffer at pH = 6.0 for 5 minutes in pressure cooker at boil, or
   - pronase E at 1 mg/ml in PBS for 2 minutes

4. Block endogenous peroxidase

   - 0.1% sodium azide/3% hydrogen peroxide in automation buffer for 15 minutes

5. Block non-specific protein (antibody) binding

   - 10% chicken ovalbumin in Tris saline for 10 minutes

6. Primary antibody

   - 2 hours to overnight (depending on antibody) at predetermined concentration in diluent buffer

7. Linking antibody (Dako, Carpinteria, CA)

   - biotinylated anti-mouse or anti-rabbit antibody at 1:100 in diluent buffer for 1 hour

8. Streptavidin-peroxidase conjugate (Dako, Carpinteria, CA)

   - 1:100 in diluent buffer for 1 hour

9. Chromogen

   - diaminobenzidine at 1 mg/ml in automation buffer with 1 µl H_2O_2 for 30 seconds to 10 minutes

10. Signal intensification

   - 0.2% osmium tetraoxide in PBS for 1 to 10 minutes

11. Counterstain

   - 1% freshly prepared methyl green for 5 minutes

12. Dehydration and mounting

   - graded alcohols and xylene

13. Coverslip

   - cover section with mounting media, Permount (electron Microscopy Sciences, Ft. Washington, PA) and glass coverslip

For details of procedures, see Durfee, T. et al. 1994. *J. Cell Biology* 127:609-622; Allred, D.C. et al. 1993. *J. Natl Cancer Inst* 85:200-206; Oesterreich, S. et al. 1996. *Clin Cancer Res* 2:1199-1206; Diab, S.G. et al. 1997. *Breast Cancer Res Treat* 43:99-103; Makris, A. et al. 1997. *Breast Cancer Res Treat* 44:65-74; Berardo, M. et al. 1998. *Cancer* 82:1296-1302; Allred, D.C. et al. 1993. *J Histotechnol* 16:117-120.

Expected Results:

From these experiments, we will complete out initial analysis of ER-β monoclonal antibodies. Using western blot analysis, we will eliminate antibodies that have nonspecific cross reactivity with other cellular proteins isoforms and ER-α and would give anomalous results for cellular localization and in immunohistochemistry. The preliminary analysis using immunofluorescence and immunohistochemistry using tissue culture cell lines allows the determination to be made of the potential usefulness of this technique for analysis of tumor samples and the sensitivity and specificity of each antibody.

Measuring ER-beta isoforms in archived breast tumor samples

To measure levels of the ER-beta isoforms in 200 human breast tumor samples from the Baylor SPORE Breast Cancer Developmental Bank, the following steps will be taken. It will be necessary to determine how the levels and/or cellular localization (cytoplasmic vs. nuclear) correlate with other prognostic indicators, including ER, PgR, DNA ploidy, S-phase fraction, p53, HER-2/*neu*,

or histologic grade. Currently, there are several types of markers that are used to make management and treatment decisions for patients with primary breast cancer. Prognostic factors are used to estimate the chances of disease recurrence if no systemic adjuvant therapy is given. Examples of prognostic factors are axillary lymph node status, tumor size, histologic grade, and steroid receptor status. Patients with a high estimated chance of disease recurrence more often receive systemic adjuvant therapy following local surgery. Predictive factors are used to select optimal therapies for individual patients.

Examples of predictive factors are steroid receptors, S-phase by DNA flow cytometry, and, more recently, HER-2/*neu* status (Allred, D.C. et al. 1998. *Modern Pathology* 11(2):155-168). Other newer markers such as heat shock proteins Oesterreich, S. et al. 1996. *Clin Cancer Res* 2:1199-1206; Elledge, R.M. et al. 1994. *Cancer Research* 54:3752-3757), p53 (Allred, D.C. et al. 1998. *Modern Pathology* 11(2):155-168), and apoptosis (Diaz-Cano, S.J. et al. 1997. *Diagn. Mol. Path.* 6:199-208) are not yet used routinely in the clinic, but they have provided important information about the biology of breast cancer, and their prognostic and predictive abilities are currently being evaluated.

ER-beta protein isoforms, which are involved in estrogen signal transduction and transcriptional regulation, may also be important prognostic and/or predictive markers for breast cancer and other types of cancer. To address this issue definitively, it will be necessary to correlate the level of these proteins with the clinical outcomes of patients with breast cancer. Required first steps before such definitive studies can be designed and conducted are to obtain preliminary information about the levels of these proteins that are present in human breast tumors, to determine

the variability of expression of these factors among tumors, to determine how often these factors are present in the cytoplasm or the nucleus, and to determine if the levels or the localization correlate other established prognostic or predictive factors. Since no correlation has been reported between expression and tumor grade and alteration in cellular localization, differential expression of the levels and localization among tumors may be seen. Associations with other prognostic factors that are known to correlate with tumor grade (e.g., steroid receptor status and S-phase fraction) may also be seen. The magnitude and variability of the expression of the ER-β isoforms and the correlation with other factors will greatly influence sample size requirements for subsequent correlative studies.

To address these issues, IHC will be used to test the antibodies of the invention for determining the levels and cellular localization in tumor samples from the Baylor SPORE Breast Cancer Development Bank. Many other factors, including ER, PgR, DNA ploidy, S-phase faction, p53, HER-2/*neu*, and histogolic grade have already been measured in these tumors, but no clinical follow-up is available for any of these patients. Results of these studies will be used to determine which proteins will be best for further study. These results will also be used to refine the sample size requirements for the follow-up study.

Methods.

Immunohistochemistry and Analysis of Protein Levels.

The levels of ER-βeta isoform specific antibodies will be determined by IHC using procedures previously described. ER levels and PgR levels have been previously determined by

biochemical assays (Dressler, L.G. et al. 1988. *Cancer* 61:420-427) and by IHC using the 6F11 antibody from Novocastra for ER (Harvey, J.M. et al. *J Clin Oncol* (in press)) and the KD68 antibody from Abbott Laboratory for PgR (6156); DNA ploidy and S-phase fraction have been previously determined by flow cytometry. (Dressler, L.G. et al. 1988. *Cancer* 61:420-427, Wenger, C.R. et al. 1993. *Breast Cancer Res Treat* 28:9-20); p53 has been measured by IHC using a cocktail of antibodies that includes Pab 1801 and Pab 240 from GeneTex (Allred, D.C. et al. 1993. *J Natl Cancer Inst* 85:200-206), and HER-2/*neu* has been previously measured by IHC using the TAB 250 antibody from Triton (Elledge, R.M. et al. 1998. *Clin Cancer Res* 4:7-12). Immunohistochemistry procedures will be as previously described.

In addition to the information and expression levels detected using IHC, the levels of protein will be determined by western blotting an/or immunoprecipitation/western blotting techniques. In these procedures pulverized samples of the identical tumor that was used in IHC will be lysed in 5% SDS, and the protein concentration will be quantitated using BCA reagent assay (Pierce, Rockford, IL). These levels can then be correlated to the scoring of the IHC and to the expression of the other predictive markers that have previously been documented from these tumor samples.

Statistical analyses

Each protein's levels will first be analyzed as continuous variables. For prognostic factors that are measured on a continuous scale (ER, PgR, S-phase fraction, p53, HER-2/*neu*), associations with each protein will be assessed by Spearman non-parametric correlation coefficients. For factors

that are inherently dichotomous or are usually classified into two categories (DNA ploidy, ER status, PgR status, p53 status, HER-2/*neu* status), Wilcoxon non-parametric rank sum tests will be used to compare protein levels. The distributions of IHC scores for each protein will then be examined to see if there any natural breakpoints that might be used to differentiate low levels from high levels, and appropriate procedures for binary variables (Chi-square tests, Fisher exact tests) will be applied. With a sample of 200 specimens, the width of a 95% confidence interval for rate of over-expression of each protein will be less than 14%. If the data follow normal distributions or can be transformed to normal distributions, an 80% power to detect a correlation of 0.195 and 90% to detect a correlation of 0.224 with any of the standard prognostic factors is predicted.

What is claimed is:

1. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein.

2. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein in a histological sample.

3. The monoclonal antibody of claim 2 wherein said histological sample is a fixed paraffin-embedded tissue.

4. The monoclonal antibody of claim 3 wherein said tissue is selected of ERB-containing tissues selected from the group of breast, prostate, testis, ovary, thymus, spleen, adipose, uterus, pituitary, and kidney.

5. The monoclonal antibody of either of claims 1 or 2 wherein said epitope lies within amino acid residues 1-146 of ERB.

6. The antibody of claim 5, said antibody consisting of anti-estrogen receptor-β antibody produced by hybridoma clone 14C8.

7. The monoclonal antibody of either of claims 1 or 2 wherein said epitope lies within amino acid residues 1-36 of ERB.

8. The monoclonal antibody of either of claim 1 or claim 2 wherein said epitope lies within amino acid residues 36-146.

9. The monoclonal antibody of either of claim 1 or claim 2 where in said estrogen receptor-β protein is an isoform selected from the group ERβ1, ERβ2, ERβ3, ERβ4, ERβ5, and Erβcx.

10. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein, wherein said epitope is selected from the group of epitopes lying within the amino acid ranges 1-146, 1-36 and 36-146.

11. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein in a histological sample, wherein said histological sample is a fixed paraffin-embedded tissue.

12. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein in a histological sample, wherein said histological sample is a fixed paraffin-embedded tissue, wherein said tissue is breast tissue.

13. A monoclonal antibody said monoclonal antibody having binding specificity for a single epitope of the estrogen receptor-β protein in a histological sample, wherein said histological sample is a fixed paraffin-embedded tissue, wherein said tissue is breast tissue, wherein said epitope is one lying within the amino acid range 1-146 of said protein, and wherein said monoclonal antibody is produced by hybridoma clone 14C8.

14. The monoclonal antibody of either of claim 1 or claim 2 selected from the group consisting of an anti-estrogen receptor-β antibody from hybridoma clones 14C8, 14G2, 4D2, 6B12, and 6A12.

15. A monoclonal antibody selected from the group consisting of anti-estrogen receptor-β antibodies from hybridoma clones 14C8, 14G2, 4D2, 6B12, and 6A12.

16. A hybridoma which produces a monoclonal antibody that specifically binds to estrogen receptor-β, said hybridoma comprising:

an antibody producing cell producing a monoclonal antibody having binding specificity for a single epitope on estrogen receptor-β protein; and

a tumor cell fused with said antibody producing cell.

17. A method for detection of estrogen receptor-β in a biological sample comprising:

contacting said sample with the antibody of claim 1; and

detecting the presence of estrogen receptor-β in said sample using an analytical technique selected from the group consisting of radioimmunoassay, immunoprecipitation, western blotting, enzyme-linked immunosorbent assays (ELISA), and immunocytochemistry.

18. A method for detection of a range of estrogen receptor-β isoforms in a biological sample comprising:

differentially labeling at least two antibodies of claim 9, each antibody specifically and uniquely recognizing one of said isoforms

contacting said sample with the antibodies; and

detecting the presence of estrogen receptor-β isoforms in said sample using an analytical technique selected from the group consisting of radioimmunoassay, immunoprecipitation, Western blotting, enzyme-linked immunosorbent assays (ELISA), and immunocytochemistry.

19. A kit for detection of estrogen receptor-β in a biological sample comprising:

a blocking reagent;

a monoclonal antibody of either of claim 1, claim 2 or claim 9;

a secondary antibody;

an avidin solution;

a biotinylated horseradish peroxidase solution;

a hydrogen peroxide solution; and

diaminobenzidine tablets.